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Transfer of Multiple Drug Resistance Plasmids between Bacteria of Diverse Origins in Natural Microenvironments

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Plasmids harboring multiple antimicrobial-resistance determinants (R plasmids) were transferred in simulated natural microenvironments from various bacterial pathogens of human, animal, or fish origin to susceptible strains isolated from a different ecological niche. R plasmids in a strain of the human pathogen *Vibrio cholerae* O1 El Tor and a bovine *Escherichia coli* strain were conjugated to a susceptible strain of the fish pathogenic bacterium *Aeromonas salmonicida* subsp. *salmonicida* in marine water. Conjugations of R plasmids between a resistant bovine pathogenic *E. coli* strain and a susceptible *E. coli* strain of human origin were performed on a hand towel contaminated with milk from a cow with mastitis. A similar conjugation event between a resistant porcine pathogenic *E. coli* strain and a susceptible *E. coli* strain of human origin was studied in minced meat on a cutting board. Conjugation of R plasmids between a resistant strain of the fish pathogenic bacterium *A. salmonicida* subsp. *salmonicida* and a susceptible *E. coli* strain of human origin was performed in raw salmon on a cutting board. R plasmids in a strain of *A. salmonicida* subsp. *salmonicida* and a human pathogenic *E. coli* strain were conjugated to a susceptible porcine *E. coli* strain in porcine feces. Transfer of the different R plasmids was confirmed by plasmid profile analyses and determination of the resistance pattern of the transconjugants. The different R plasmids were transferred equally well under simulated natural conditions and under controlled laboratory conditions, with median conjugation frequencies ranging from 3×10^{-6} to 8×10^{-3}. The present study demonstrates that conjugation and transfer of R plasmids is a phenomenon that belongs to the environment and can occur between bacterial strains of human, animal, and fish origins that are unrelated either evolutionarily or ecologically, even in the absence of antibiotics. Consequently, the contamination of the environment with bacterial pathogens resistant to antimicrobial agents is a real threat not only as a source of disease but also as a source from which R plasmids can easily spread to other pathogens of diverse origins.

The ease with which bacteria become resistant to currently used antimicrobial agents has been and continues to be of concern to clinicians, public health officials, and researchers. Today, transferable drug resistance represents a major threat to the treatment of infectious diseases in both humans and animals, including farmed fish. The use of antimicrobial agents in both human and veterinary medicine exerts a strong selective pressure inducing resistance to antimicrobial agents among bacteria (18, 24, 26, 48). Generally, bacteria with the highest level of resistance are isolated from environments contaminated with antimicrobial agents, e.g., hospitals, fish farms, sewage effluents, and wastewater (7, 9, 11, 15, 24, 29, 32, 37, 41, 45). However, resistant bacteria have also been isolated from apparently nonselective environments (24, 29).

Many attempts have been made to show that plasmid transfer between bacteria occurs in a variety of natural habitats, e.g., wastewater (12, 29), sewage (9), seawater (11, 13, 43), river water (16), lake water (14, 34), river epilithon (3), sediments (11, 43), soil (46), gastrointestinal tracts (26, 27, 47), and growing radish plants and aqueous sawdust suspensions (44). However, most of these studies focused on environmental bacteria or pathogens belonging to the same bacterial family or group and derived from the same ecological niche. Conjugation and transfer of resistance plasmids (R plasmids) between distantly related bacteria have been described by several authors (10, 19, 30, 31), but most of these experiments have been performed in laboratories under standardized conditions.

The present work was performed in order to study transfer of R plasmids between pathogenic bacteria of diverse origins under simulated natural conditions and to compare these frequencies of transfer with the results obtained under standardized laboratory conditions. Different experiments on conjugation between a bacterial pathogen of human, animal, or fish origin harboring a multiple drug resistance plasmid and a susceptible strain from another ecological niche were performed. The bacterial strains and R plasmids included are well characterized and thoroughly studied under controlled laboratory conditions, but knowledge about transfer of these R plasmids under natural circumstances is unavailable. The present experiments were performed in simulated natural microenvironments and designed to illustrate different everyday situations that represent potential sources for transfer of such R plasmids. Seawater containing a susceptible fish pathogen was contaminated with a resistant human or animal pathogen in order to study if bacteria of human or animal origin might represent a source of R plasmids for fish pathogens. Bovine milk containing a resistant mastitis pathogen was splashed onto a hand towel preincubated with susceptible bacteria of human origin to illustrate what might happen when a farmer uses a towel after milking a cow with mastitis. Minced meat containing a resistant porcine pathogen was prepared on a cutting board preincubated with susceptible bacteria of human origin in order to study if transfer of R plasmids might occur from bacteria in meat to human bacterial strains during food preparation. Also, a salmon contaminated with a resistant fish pathogen was brought into contact with susceptible bacte-

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TABLE 1. Donor strains, recipient strains, and transconjugants

Bacterium	Environment	Mol wt (10^6) of plasmid(s)	Resistance determinant(s)
Donors			
E. coli NVH4061[a]		36, 120	Amp, Tet, Str, Tri, Sul
E. coli NVH4051[b]		4.0, 36	Amp, Tet, Str, Tri, Sul
E. coli NVH4083[c]		2.7, 3.2, 4.0, 100, 120	Amp, Tet, Str, Sul, Chl
V. cholerae NVH4122[d]		130	Amp, Tet, Str, Tri, Sul, Chl
A. salmonicida subsp. *salmonicida* NVH4133[e]		3.4, 3.5, 3.6, 6.5, 25, 85	Tet, Tri, Sul
Recipients			
E. coli DH5[f]			Nal
E. coli NVH1570[g]		2.2, 4.5, 105, 120	Nal
E. coli NVH1535[h]		6, 30, 40, 80	Nal
A. salmonicida subsp. *salmonicida* AL2027[i]		3.4, 3.5, 3.6, 4.2, 52	Nal
Transconjugants (donors)			
E. coli DH5 (*E. coli* NVH4061)	Hand towel	**36**	Nal, Amp, Tet, Str, Tri, Sul
E. coli DH5 (*E. coli* NVH4051)	Minced meat	**4.0, 36**	Nal, Amp, Tet, Str, Tri, Sul
		25	Nal, Amp, Tet, Tri
E. coli DH5 (*A. salmonicida* subsp. *salmonicida* NVH4133)	Raw salmon	**25**	Nal, Tet, Tri, Sul
E. coli NVH1570 (*E. coli* NVH4083)	Pig feces	2.2, 4.5, 105, **120**	Nal, Amp, Tet, Str, Sul, Chl
E. coli NVH1535 (*A. salmonicida* subsp. *salmonicida* NVH4133)	Pig feces	6, **25**, 30, 40, 80	Nal, Tet, Tri, Sul
A. salmonicida subsp. *salmonicida* AL2027 (*E. coli* NVH4061)	Seawater	3.4, 3.5, 3.6, 4.2, **36**, 52	Nal, Amp, Tet, Str, Tri, Sul
A. salmonicida subsp. *salmonicida* AL2027 (*V. cholerae* NVH4122)	Seawater	3.4, 3.5, 3.6, 4.2, 52, **130**	Nal, Amp, Tet, Str, Tri, Sul, Chl

[a] Isolated at the Norwegian College of Veterinary Medicine in 1992 from a cow with mastitis.
[b] Isolated at the Norwegian College of Veterinary Medicine in 1992 from a piglet with diarrhea.
[c] Isolated at Ullevål Hospital, Oslo, Norway, in 1992 from a patient with a urinary tract infection.
[d] Isolated in Kampala, Uganda, in 1992 from a patient with cholera (22).
[e] Isolated in Norway in 1991 from an Atlantic salmon with furunculosis.
[f] Chromosomally resistant to nalidixic acid (17); contains no plasmids.
[g] Isolated at the Norwegian College of Veterinary Medicine in 1962 from a healthy Norwegian Landrace pig. The strain is hemolytic, expresses the fimbrial antigen F5 (K99), and belongs to serogroup O141.
[h] Isolated at the Norwegian College of Veterinary Medicine in 1962 from a Norwegian Landrace pig with edema disease. The strain is hemolytic and F5 positive.
[i] Isolated at Apothekernes Laboratorium A/S, Oslo, Norway, from a salmon with furunculosis.

ria of human origin on a cutting board to investigate the possibilities of transfer of R plasmids from bacteria colonizing fish intended for consumption to human bacterial strains during food preparation in the kitchen. Feces from a pig containing a susceptible porcine pathogen were contaminated with resistant fish or human pathogens to evaluate if such contamination might represent a source of R plasmids for porcine bacteria.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used as either donors or recipients are listed in Table 1. Both among donors and among recipients, bacterial strains of human, animal, and fish origins were included. The plasmid contents and the antimicrobial susceptibility patterns of the strains are also listed in Table 1. *Escherichia coli* NVH1570 and NVH1535 were initially susceptible to all antimicrobial agents tested but were made resistant to nalidixic acid (NAL) by cultivation in increasing concentrations of the agent.

Susceptibility testing. Donors, recipients, and transconjugants were tested for their antimicrobial susceptibilities in a standard agar disk diffusion test as previously described (21) by using Mueller-Hinton (MH) agar plates (Bacto; Difco Laboratories, Detroit, Mich.) and Neo-Sensitabs (A/S Rosco, Taastrup, Denmark). The respective discs used contained NAL (130 μg), ampicillin (AMP) (30 μg), tetracycline (TET) (80 μg), streptomycin (STR) (100 μg), trimethoprim (TRI) (5.2 μg), sulfonamides (SUL) (240 μg), and chloramphenicol (CHL) (60 μg). The results were interpreted as recommended by the Reference Group for Antibiotics in Sweden (6a).

Plasmid isolation. Plasmids were isolated by the method of Birnboim and Doly (5) and separated by vertical electrophoresis at 130 V for 4 h using 1.0% (wt/vol) agarose gels (SeaKem GTG; FMC BioProducts, Rockland, Maine) and TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA). Gels were stained with ethidium bromide, and photographs were taken under UV transillumination. Plasmids were routinely prepared from all strains involved in the conjugation experiments.

Conjugation experiments. NAL-resistant recipients were used in all matings in order to have a selectable marker for selection against the donor. Resistance to TET was used as a selection marker to select transconjugants from recipients. To control for recipient mutations, unmated recipient strains were also spread directly onto selective plates containing TET.

To compare the results from conjugation experiments under simulated natural microenvironments with those obtained under controlled laboratory conditions, control matings in nutrient broth were performed. Both donor and recipient strains were incubated overnight in MH broth at 37°C and diluted to approximately 6×10^8 cells per ml by using McFarland's nephelometer standard no. 2 (28). One milliliter of donor broth was mixed with 1 ml of recipient broth in 4 ml of MH broth and incubated for 24 h at room temperature. After incubation the conjugation mixtures were diluted 10-fold in 0.9% saline to a 10^{-7} dilution. Samples (0.1 ml) from each dilution were spread onto one MH agar plate containing 20 μg

of NAL per ml (NAL-agar plate) to select recipients and one MH agar plate containing 20 μg of NAL and 10 μg of TET per ml (TET-agar plate) to select transconjugants. Colonies were counted after overnight incubation at 37°C in a 5% CO_2 atmosphere (48 h aerobically at 20°C for conjugations involving recipients of *Aeromonas salmonicida* subsp. *salmonicida*. Transfer frequency was estimated by dividing the number of transconjugants per milliliter by the number of recipients per milliliter in the mating mixture.

For the experiment on conjugation in raw salmon, comparative controls were performed on an MH agar plate, because the R plasmid of *A. salmonicida* subsp. *salmonicida* NVH4133 is more efficiently transferred on a solid medium than in liquid medium (42). Three to five colonies of both the donor strain and the recipient strain were mixed on an MH agar plate and incubated for 24 h at room temperature. The further steps of the procedure were performed as described for conjugation in MH broth.

Several transfer experiments were performed under conditions intended to mimic natural environments that may present opportunities for conjugation. In all matings, overnight cultures (MH broth) of both donor and recipient strains diluted to approximately 6×10^8 cells per ml (McFarland 2) were used. To evaluate the stability of the R plasmids in the transconjugants obtained, the different types of transconjugants were susceptibility tested after 10 passages on a blood agar plate. The designs of the conjugation experiments differed (see below), but the procedure after the mating was performed as described for the controls.

(i) Conjugation in seawater. *A. salmonicida* subsp. *salmonicida* AL2027 served as the recipient, while either the human pathogenic strain *Vibrio cholerae* NVH4122 or the animal pathogenic strain *E. coli* NVH4061 was used as the donor. For conjugation medium, newly collected seawater from the Oslo fjord in Norway was chosen. The salinity of the water was 2.1% NaCl, measured by method 89 of the Nordic Committee on Food Analysis (33a). The following quality factors of the water were measured by the Norwegian Standard (NS) methods: 50 μg of P per liter (NS 4725), 90 mg of $KMnO_4$ per liter (NS 4732), 200 mS/cm (NS 4721), 241 degrees of hardness in water (°dH) (42.9 mmol of Ca^{2+} plus Mg^{2+} per liter (NS 4728), 0.07 ppm of Cu (NS 4772 to 4774 and 4777), 2.0 ppb of Pb (NS 4772 to 4774 and 4777), and no detectable amount of Mn, Cd, or Fe (NS 4772 to 4774 and 4777). Water samples of 100 μl were spread onto a NAL-agar plate to control for growth of NAL-resistant coliform bacteria, onto a blood agar plate to get an impression of the general flora in the water, and onto a MacConkey agar plate together with Neo-Sensitabs (TRI, SUL, AMP, TET, STR, and CHL) to control for the presence of antibiotic-resistant coliform bacteria.

The pellet from 0.5, 1.0, or 5.0 ml of MH broth (McFarland 2) was resuspended in 5 or 10 ml of seawater, producing concentrations of both donor and recipient of 3×10^7, 6×10^7, 1.2×10^8, and 6×10^8 cells per ml of seawater. Parallel mixtures were incubated for 24 h at 6 or 20°C.

The above-described experiments with seawater were repeated with a subinhibitory concentration of 0.5 or 0.25 μg of TET per ml of seawater for *V. cholerae* and 0.5 or 1.0 μg of TET per ml of seawater for *E. coli*.

(ii) Conjugation on a hand towel. For a donor strain, *E. coli* NVH4061 isolated from a cow with mastitis was chosen, while *E. coli* DH5 served as a recipient strain. Conjugation was performed at room temperature on the surface of a cotton towel. The pellet from 4 ml of the MH broth (McFarland 2) of the donor strain was added to 50 ml of either fresh cow's milk or pasteurized and homogenized cow's milk, producing a concentration of 5×10^7 donor cells per ml of milk. This mixture was preincubated at 37°C for 1 h. A hand towel (1,750 cm^2) made of 100% cotton was preincubated with 50 ml of 0.9% saline containing a total of 6×10^8 *E. coli* DH5 cells for 1 h at room temperature. After preincubation with *E. coli* DH5, the contaminated milk was spread onto and rubbed into the towel. The towel was incubated at room temperature.

After 1 and 24 h, 10 g of the towel was mixed with 9 ml of 0.9% saline, this mixture was further diluted 10-fold, and samples (0.1 ml) from the different dilutions were spread onto selective agar plates as previously described.

Untreated milk samples were controlled for the presence of bacteria by inoculating milk on blood agar plates and incubating the plates at 37°C in a CO_2 atmosphere.

(iii) Conjugation in minced meat on a cutting board. *E. coli* NVH4051 and *E. coli* DH5 were used as the donor strain and the recipient strain, respectively. Conjugation was performed in minced meat, first on a wooden cutting board (375 cm^2) at room temperature and then in a refrigerator at 6°C.

A 4.0- or 8.0-ml sample of a donor MH broth (McFarland 2) or the pellet of 100 ml of a donor MH broth (McFarland 2) resuspended in 10 ml of MH broth was added to 100 g of fresh minced meat from cattle and swine, producing concentrations of 2.4×10^7, 4.8×10^7, and 6.0×10^8 donor cells per ml of meat, respectively. The bacteria were mixed well into the meat, and the mixture was left at room temperature for 1 h. A 4.0- or 8.0-ml sample of recipient broth or the pellet of 100 ml of recipient broth resuspended in 10 ml of MH broth (2.4×10^9, 4.8×10^9, and 6.0×10^{10} bacteria, respectively) was spread onto a wooden cutting board, and the board was preincubated for 1 h at room temperature. The meat was then kneaded on the board for 2 min together with 18 ml of saline. After a 1-h incubation at room temperature, the meat was placed in the refrigerator at 6°C for 23 h. Before and after 23 h in the refrigerator, a sample of 10 g of minced meat was dissolved in 9 ml of saline. One milliliter of this mixture was diluted 10-fold, and samples from each dilution were plated on selective agar plates and incubated as previously described. After removal of the meat, 9 ml of saline was mixed with the remnants on the board. One milliliter of this mixture was diluted and used for plating as described elsewhere. The board was then left at room temperature for 23 h before the same procedure was repeated.

The initial bacterial content of the minced meat was controlled by culturing a sample on a blood agar plate to get an impression of the general flora, on a NAL-agar plate to control for growth of NAL-resistant coliform bacteria, and on an MH agar plate together with Neo-Sensitabs (TRI, SUL, AMP, TET, STR, and CHL) to control for the presence of drug-resistant coliform bacteria.

(iv) Conjugation in raw salmon on a cutting board. Ten small Atlantic salmon (*Salmo salar* L.), about 50 g each, were intraperitoneally injected with a fresh broth of *A. salmonicida* subsp. *salmonicida* NVH4133 and put into an aquarium with fresh water, at 6°C, together with 10 untreated similar-size salmon. At day 3 one fish died from furunculosis. To verify the diagnosis, samples from the pronephros and from an abscess on the tail were cultured by using blood agar and NAL-agar plates and incubation at 20°C for 2 days, and susceptibility testing was performed thereafter. The fish was prepared on a plastic cutting board (264 cm^2) inoculated with 7×10^9 *E. coli* DH5 cells. After incubation on the board for 1 h at 20°C, 10 g of the fish material was mixed with 9 ml of 0.9% saline and diluted 10-fold in saline. Samples of 0.1 ml were inoculated on selective plates and incubated as previously described. The remains of the fish were put in the refrigerator at 6°C

overnight. The next day 10 g of the fish material was diluted 10-fold in saline, and samples were plated and incubated as previously described.

As a negative control, untreated healthy salmon was prepared on a cutting board inoculated with *E. coli* DH5 as described for the fish with furunculosis.

(v) Conjugation in feces from a pig. Fresh feces from a slaughtered healthy pig that had never received antimicrobial treatment were collected. One gram of feces was diluted in 9 ml of 0.9% saline. Of this mixture, 100 μl was spread onto a NAL-agar plate to control for growth of NAL-resistant coliform bacteria, onto a blood agar plate to get an impression of the general flora in the feces, and onto a MacConkey agar plate together with Neo-Sensitabs (TRI, SUL, AMP, TET, STR, and CHL) to control for the presence of antibiotic-resistant coliforms. One hundred grams of feces was added to the pellet from 10 ml of MH broth (McFarland 2) containing *E. coli* NVH1570 and preincubated for 1 h at 20°C. Then the pellet of 10 ml of MH broth (McFarland 2) containing *E. coli* NVH4083 was added. The mixture was well blended and incubated at 20°C. After 1 and 24 h, 10 g of feces was diluted 10-fold in 0.9% saline and samples were plated and incubated as previously described.

The above-described experiment was also performed with *E. coli* NVH1535 as the recipient and *A. salmonicida* subsp. *salmonicida* NVH4133 as the donor.

RESULTS

Conjugation experiments in general. Table 2 shows the median frequencies of transfer and the ranges of frequencies for the different conjugation experiments at various incubation temperatures and periods. The frequencies of transfer observed for control conjugations in MH media at 20°C for 24 h are also presented in Table 2.

The calculated frequencies of transfer within the different sets of conjugation experiments under conditions intended to mimic natural environments were of the same order of magnitude as the frequencies measured under standard laboratory cultural conditions. Variations in either temperature or conjugation time did not influence the frequencies observed.

Antimicrobial susceptibility testing and plasmid profile analyses (Table 1) revealed that the transconjugants expressed the same resistance patterns (in addition to NAL resistance) as the donor strains and confirmed the transfer of an R plasmid from the donor to the recipient (boldfaced).

Susceptibility testing of the different types of transconjugants after 10 passages on a blood agar plate revealed that the transconjugants still expressed the same resistance pattern, indicating that the plasmids transferred were stably inherited.

(i) Conjugation in seawater. Culturing from untreated water samples gave rise to growth of several bacterial species, mainly gram-positive bacteria. However, no *A. salmonicida* subsp. *salmonicida* bacteria nor bacteria resistant to the antimicrobial agents tested could be detected. Hence, the multiple drug resistant *A. salmonicida* subsp. *salmonicida* bacteria detected after accomplishment of the conjugation experiments are believed to be true transconjugants containing the R plasmids from the donor strains added.

Contemporaneous changing of the concentration of the donor and the concentration of the recipient did not affect the frequencies measured. Using gradually lower concentrations only resulted in problems of detecting transconjugants because of fewer recipients. The efficiency of transfer was not temperature dependent for the bacteria and temperatures studied. However, adding a subinhibitory concentration of TET resulted in an increase of transfer of antimicrobial resistance from *V. cholerae* to *A. salmonicida* subsp. *salmonicida* at 20°C, whereas no effect of adding TET on the transfer from *E. coli* NVH4061 to *A. salmonicida* subsp. *salmonicida* could be detected.

(ii) Conjugation on a hand towel. Culturing from untreated milk samples did not result in bacterial growth, thus simplifying the interpretation of the findings on the selection plates. The calculated frequencies of plasmid transfer were not influenced by the type of milk used (either fresh cow's milk or pasteurized and homogenized cow's milk).

(iii) Conjugation in minced meat on a cutting board. Culturing from untreated samples of minced meat gave rise to 400 to 1,000 coliform bacteria per g, a concentration considered acceptable for food. No coliform bacteria harboring resistance to the antimicrobial agents in question were detected, suggesting that the transconjugants observed after conjugation could not have been derived from these bacteria nor could the R plasmids in the transconjugants have originated from these bacteria.

The mean frequencies of transfer in minced meat and in the remnants on the cutting board were of the same order of magnitude, both after 1 h and after 24 h. However, some experiments did not give rise to detectable numbers of transconjugants. This refers to eight instances of transfer in the remnants on the cutting board (four after 1 h and four after 24 h), two instances of transfer in minced meat after 1 h, and one instance of transfer in minced meat after 24 h of conjugation. In these cases the readings of the recipients were of a magnitude (10^4) which could hardly result in detectable transconjugants, taking into consideration a frequency of transfer of 10^{-5}. It was the experiments with the lowest concentrations of bacteria which resulted in this phenomenon. Fewer transconjugants and recipients were found on the cutting board than in the minced meat, with the differences ranging from negligible to 1 in 10^4. Also, fewer recipients were detected per gram of meat, compared with the amounts added to the meat, with the differences ranging from 1 in 6 to 1 in 10^3.

A total of 36 minced-meat transconjugants were tested for antimicrobial susceptibility. Of these transconjugants, 75% were resistant only to NAL, AMP, TET, and TRI whereas 25% expressed all resistance determinants harbored by the donor strain. The latter type of transconjugants showed the same plasmid content as the donor strain (4 and 36 MDa), while the transconjugants lacking some of the determinants contained a 25-MDa R plasmid, indicating that only a part of the R plasmid had been transferred (Table 1).

(iv) Conjugation in raw salmon on a cutting board. This experiment was performed only once because of the lack of naturally infected salmon and problems with artificial infection of the salmon. From the only salmon suspected of having furunculosis and thus included in the conjugation study, *A. salmonicida* subsp. *salmonicida* NVH4133 was isolated in pure culture from an abscess and from the pronephros. The frequency of plasmid transfer in this artificially infected salmon was similar to the corresponding frequency of transfer on an MH agar plate. No bacterial growth from the control fish was observed, and no transconjugants were detected after the preparation of these fish on a cutting board preincubated with *E. coli* DH5.

(v) Conjugation experiments in feces from a pig. The control plates incubated aerobically overnight at 37°C did not result in growth of any hemolytic bacteria or NAL-resistant bacteria. On the other hand, the MacConkey agar plate showed growth of coliform bacteria resistant to STR, but these colonies were susceptible to NAL. The transconjugants were hemolytic when

TABLE 2. R-plasmid transfer under simulated natural conditions and under standardized laboratory conditions

Mating pair[a] (environment)	Transfer frequency(ies)			
	Simulated environment			MH broth
	Median	Range	No. of expts	
A (seawater)				
Without TET				
6°C, 24 h	5×10^{-5}	5×10^{-6} to 1×10^{-4}	6	1×10^{-6} and 1×10^{-6}
20°C, 24 h	2×10^{-5}	6×10^{-6} to 2×10^{-5}	6	5×10^{-5} and 2×10^{-5}
With TET				
6°C, 24 h	5×10^{-5}	2×10^{-5} to 1×10^{-4}	5	3×10^{-4} and 5×10^{-4}
20°C, 24 h	3×10^{-5}	4×10^{-6} to 5×10^{-5}	4	1×10^{-5}
B (seawater)				
Without TET				
6°C, 24 h	4×10^{-5}	1×10^{-5} to 1×10^{-4}	6	5×10^{-4}
20°C, 24 h	2×10^{-5}	5×10^{-6} to 7×10^{-5}	7	5×10^{-5}
With TET				
6°C, 24 h	4×10^{-6}	3×10^{-7} to 1×10^{-5}	7	5×10^{-4} and 7×10^{-3}
20°C, 24 h	3×10^{-3}	4×10^{-4} to 5×10^{-3}	5	6×10^{-1}
C (hand towel with milk)				
20°C, 1 h	3×10^{-4}	1×10^{-4} to 1×10^{-3}	4	
20°C, 24 h	1×10^{-3}	3×10^{-4} to 2×10^{-3}	4	1×10^{-3} and 2×10^{-4}
D (minced meat)				
20°C, 1 h	3×10^{-5}	1×10^{-6} to 1×10^{-4}	5	
20 + 6°C, 1 + 23 h	3×10^{-5}	1×10^{-6} to 2×10^{-4}	5	
D (cutting board)				
20°C, 1 h	4×10^{-5}	1×10^{-5} to 6×10^{-5}	2	
20°C, 24 h	3×10^{-5}	5×10^{-6} to 5×10^{-5}	2	1×10^{-5} and 2×10^{-5}
E (raw salmon), 20°C, 24 h	8×10^{-3}		1	6×10^{-3} and 5×10^{-4}[b]
F (pig feces)				
20°C, 1 h	6×10^{-6}	2×10^{-6} to 1×10^{-5}	2	
20°C, 24 h	2×10^{-5}	1×10^{-5} to 3×10^{-5}	2	7×10^{-5}
G (pig feces)				
20°C, 1 h	3×10^{-6}		1	
20°C, 24 h	3×10^{-6}		1	2×10^{-6}

[a] A, bovine pathogenic *E. coli* NVH4061 (donor) and fish pathogenic *A. salmonicida* subsp. *salmonicida* AL2027 (recipient); B, human pathogenic *V. cholerae* NVH4122 (donor) and fish pathogenic *A. salmonicida* subsp. *salmonicida* AL2027 (recipient); C, bovine pathogenic *E. coli* NVH4061 (donor) and *E. coli* DH5 of human origin (recipient); D, porcine pathogenic *E. coli* NVH4051 (donor) and *E. coli* DH5 of human origin (recipient); E, fish pathogenic *A. salmonicida* subsp. *salmonicida* NVH4133 (donor) and *E. coli* DH5 of human origin (recipient); F, human pathogenic *E. coli* NVH4083 (donor) and porcine pathogenic *E. coli* NVH1570 (recipient); G, fish pathogenic *A. salmonicida* subsp. *salmonicida* NVH4133 (donor) and porcine pathogenic *E. coli* NVH1535 (recipient).

[b] An MH agar plate was used.

grown on a blood agar plate, and they expressed the same antimicrobial resistance patterns (in addition to NAL resistance) as the donor strains (Table 1).

Plasmid profile analyses (Table 1) revealed that the *E. coli* NVH1535 transconjugants had received a 25-MDa R plasmid from *A. salmonicida* subsp. *salmonicida* NVH4133. For the *E. coli* NVH1570 transconjugants, no additional plasmid could be detected. However, *E. coli* NVH1570 possesses a plasmid of the same size (120 MDa) as one of the plasmids in the donor strain, *E. coli* NVH4083, and consequently, these two plasmids cannot be differentiated in a plasmid profile analysis. The fact that the transconjugants showed the same resistance pattern as the donor strain and the absence of a plasmid different from the plasmids in the recipients indicates that *E. coli* NVH4083 rather than some other strain initially present in feces was the real donor and that a 120-MDa R plasmid had been transferred from it.

DISCUSSION

The frequencies of bacterial strains resistant to antimicrobial agents have increased dramatically in the environment as

a consequence of the widespread use of these drugs (4, 18, 24, 26, 48). As transfer of plasmid-encoded resistance to antimicrobial agents is a significant public health concern, the possibility of transfer of resistance genes between bacteria in natural habitats has attracted attention. While previous studies involving plasmid transfer under natural conditions were carried out between bacteria that were related either evolutionarily or ecologically, this work examines the possibilities of gene transfer between distantly related species or microorganisms that are normally widely separated in their natural habitats. The experimental conditions were designed to mimic natural environments that may present opportunities for conjugation. Although selected bacterial strains were studied, the experiments illustrate the possibilities of spread of R plasmids in the environment between bacteria of diverse origins.

The finding that the R plasmids studied were transferred between pathogenic bacteria of diverse origins under simulated natural conditions with frequencies of the same order of magnitude as under standard laboratory cultural conditions is most noteworthy. Conjugation in the absence of traditional media, such as in the experiment with the hand towel, illustrates remarkably well that R-plasmid transfer is a phenomenon that belongs to the environment and is not restricted to laboratory conditions. Our results support the notion that bacteria are able to transfer drug resistance in a nonoptimal environment such as during starvation in various aquatic environments (6, 8, 11–13, 48).

Transfer of R plasmids was shown to occur in feces from a pig, suggesting that such transfer might also occur in the gut. However, several authors report that facultative anaerobe bacteria do not readily transfer in the gut except during antimicrobial treatment (1, 2, 47), because of inhibition by the presence of fatty acids (47) or dense cultures of *Bacteroides fragilis* (1). On the other hand, food-producing animals often receive antimicrobial treatment which favors transfer of resistance. Moreover, although conjugation between facultative anaerobes in the gut is inhibited, transconjugants emerging outside the alimentary tract may subsequently colonize the gut (26, 27).

In one of the experiments performed in marine water, the addition of a subinhibitory concentration of TET increased the frequency of transfer. A stimulatory effect of TET upon conjugal transfer between certain gram-positive bacteria has been reported (39), and several epidemiological studies show an association between the use of antimicrobial agents and the development of antimicrobial resistance. Although the presence of antimicrobial agents may enhance transfer of R plasmids, a more important finding in this study is the ease of transfer of R plasmids even in the absence of such drugs.

The difficulty in detecting transconjugants, and the majority of recipients as well, on the wooden cutting board in experiments on conjugation in minced meat is in accordance with a recent report about wooden materials being bactericidal (36). If wooden materials destroy most bacteria, it may explain why so few bacteria were recovered from the cutting board.

Besides demonstrating that R plasmids transfer under highly various environments resembling natural conditions just as well as in the laboratory, the present study also supports a theory that no strong distinctions between plasmids derived from bacteria of human, animal, and fish origins exist. Many previous in vitro studies have demonstrated transfer of R plasmids from bacteria of animal (18, 48) or fish (37, 45) origin to human bacterial strains and from human pathogens to fish pathogens (22, 30, 37). Moreover, the same plasmid-bearing strains of *E. coli* may colonize both humans and animals (25). Antimicrobial-agent-resistant *E. coli* of animal origin has been proposed to constitute an important potential source of R plasmids for indigenous *E. coli* in the human gut and, subsequently, for human pathogens (26, 40). In fact, apparently related plasmids have been found in epidemiologically unrelated *E. coli* isolates from humans and pigs (20, 33). The present work demonstrates that R plasmids can spread among bacterial strains of human, animal, and fish origins that are unrelated either evolutionarily or ecologically, even under simulated natural conditions in the absence of antimicrobial agents. Thus, irrespective of where they first happen to be seen, these promiscuous plasmids seem to be drawn from a common pool and able to spread in most directions in the microbial world. Consequently, resistant bacterial strains derived from a specific ecological niche may indeed represent a source of R plasmids for other distantly related bacterial strains derived from a different ecological niche.

In the present work, conjugation as a mechanism of transfer of R plasmids between bacteria of diverse origins has been studied. Conjugation is the most studied and, probably, the most important mechanism of gene transfer among bacteria (12, 29, 46, 48). However, evidence suggesting that both transduction and transformation may be important mechanisms of gene transfer and acquisition of DNA by bacteria in aquatic environments is increasing (35, 38, 46). Transformation and transduction as, also, important mechanisms of gene transfer in the environment emphasize even more the dynamics of resistance genes in the microbial world.

The findings in the presented microcosm experiments reflect the importance of hygiene in the treatment of drinking water, in the food-processing industry, and during kitchen work and farm work. Access to water of good hygienic quality is a primary aim. The effluence of untreated wastewater should be avoided, because this contributes to the spread both of pathogenic bacterial strains and of R plasmids in the environment (26, 48). During food handling, one should follow the general guidelines for avoiding cross-contamination between different grocery products, to protect against both potential food poisoning and the spread of R plasmids. It is noteworthy that food of animal origin is not the only source of resistant bacteria, as also fruits and vegetables have been associated with resistant bacteria (23). On the farm it is of the greatest importance to avoid transporting foreign pathogens into the animal rooms by human traffic or by mixing of animals.

Not only is the ease of transfer of R plasmids in the environment a reminder for proper hygiene, but it also illustrates the importance of reducing the use of antimicrobial agents so as to decrease the level of antimicrobial resistance among bacteria. There is a general agreement that the pool of resistance genes in the environment is amplified by the use of antimicrobial agents (18, 24, 26, 48). Keeping the use of antimicrobial agents to a minimum will lower the frequencies of R plasmids among bacteria and, consequently, reduce the risk of spread of such factors in the environment, securing the continuous benefit of antimicrobial drugs.

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